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G_{12} Requirement for Thrombin-stimulated Gene Expression and DNA Synthesis in 1321N1 Astrocytoma Cells*

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Thrombin stimulation of 1321N1 astrocytoma cells leads to Ras-dependent AP-1-mediated transcriptional activation and to DNA replication. In contrast to what has been observed in most cell systems, in 1321N1 cells these responses are pertussis toxin-insensitive. The pertussis toxin-insensitive G-protein G_{12} has been implicated in cell growth and transformation in different cell systems. We have examined the potential role of this protein in AP-1-mediated transcriptional activation and DNA synthesis in 1321N1 cells. Transient expression of an activated (GTPase-deficient) mutant of $G\alpha_{12}$ increased AP-1-dependent gene expression. This response was inhibited by co-expression of a dominant negative Ala-15 Ras protein. To determine whether the pertussis toxin-insensitive G_{12} protein is involved in the thrombin-stimulated DNA synthesis, an inhibitory antibody against the C-terminal sequence of $G\alpha_{12}$ subunit was microinjected into 1321N1 cells. Microinjection of the anti-$G\alpha_{12}$ resulted in a concentration-dependent inhibition of thrombin-stimulated DNA synthesis. In contrast, microinjection of nonimmune IgG or an antibody directed against the C terminus of $G\alpha_o$ did not reduce the mitogenic response to thrombin. Furthermore, microinjection of the anti-$G\alpha_{12}$ antibody had no effect on fibroblast growth factor-stimulated DNA synthesis. These results demonstrate a specific role for $G\alpha_{12}$ in the mitogenic response to thrombin in human astroglial cells.

Thrombin is a potent mitogen for fibroblasts, astrocytes, and other cell lines (Van Obberghen-Schilling *et al.*, 1985; Cavanaugh *et al.*, 1990; Hung *et al.*, 1992; LaMorte *et al.*, 1993a, 1993b). Thrombin cleaves and activates a seven-transmembrane-spanning receptor to trigger G-protein-mediated stimulation of downstream effectors (Vu *et al.*, 1991). The actions of thrombin in 1321N1 human astrocytoma cells have been well characterized. In these cells, thrombin stimulates phospholipase C activity leading to mobilization of intracellular calcium, diglyceride generation, and redistribution of protein kinase C (Jones *et al.*, 1989; Nieto *et al.*, 1994). Thrombin receptor activation also leads to a biphasic increase in c-*jun* mRNA, an associated increase in AP-1 DNA binding activity, and a marked increase in AP-1-mediated gene expression and DNA synthesis (Trejo *et al.*, 1992; LaMorte *et al.*, 1993b). Ras function is required for the mitogenic effect of thrombin as well as thrombin-induced AP-1 transcriptional activity (LaMorte *et al.*, 1993b).

There is substantial information about the signaling pathways downstream of thrombin receptor activation; however, it is not known which of the heterotrimeric G-proteins couples thrombin to the mitogenic pathway. In fibroblasts, thrombin-stimulated DNA synthesis is sensitive to pertussis toxin (PTX)[1] (Chambard *et al.*, 1987; van Corven *et al.*, 1993). Experiments using microinjected antibodies against $G\alpha_o$ or $G\alpha_i$ suggested that the mitogenic effect of thrombin in CCL39 and in 3T3 fibroblasts is mediated through these G-proteins (LaMorte *et al.*, 1993a; Baffy *et al.*, 1994). However, in 1321N1 astrocytoma cells, thrombin-stimulated DNA synthesis and thrombin-induced gene expression are not inhibited by pertussis toxin treatment,[2] suggesting that G-proteins of the G_i/G_o family do not mediate these responses. The PTX-insensitive G-proteins G_q and/or G_{11} have also been reported to function in mitogenic signaling by thrombin and bradykinin in fibroblasts (LaMorte *et al.*, 1993a; Baffy *et al.*, 1994). However, studies comparing muscarinic and thrombin receptor signaling mechanisms in astrocytes and CCL39 fibroblasts demonstrate that, although both receptors interact with G_q/G_{11} to stimulate phospholipase C, release calcium, and activate protein kinase C, only thrombin can activate Ras and elicit cell proliferation (Trejo *et al.*, 1992; Seuwen *et al.*, 1990).[2] These data suggest that activation of G_q and phospholipase C is insufficient to account for the full mitogenic effects of thrombin and that other signaling pathways are involved.

The $G\alpha_{12}$ and $G\alpha_{13}$ subunits constitute a family of G-proteins distantly related to the other G-protein α subunits (Strathmann and Simon, 1991). Both $G\alpha_{12}$ and $G\alpha_{13}$ lack the cysteine residue that renders these proteins susceptible to ADP-ribosylation by PTX. The signaling pathways regulated by these G-proteins have not been identified. Activation of these G-proteins has nonetheless been linked to cell growth in several cell systems. Overexpression of $G\alpha_{12}$ in NIH3T3 cells leads to neoplastic transformation (Chan *et al.*, 1993), and expression of a GTPase-deficient mutant form of $G\alpha_{12}$ and $G\alpha_{13}$ can efficiently transform NIH3T3 cells (Jiang *et al.*, 1993; Xu *et*

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[1] The abbreviations used are: PTX, pertussis toxin; DMEM, Dulbecco's modified Eagle's medium; bFGF, basic fibroblast growth factor; BrdUrd, bromodeoxyuridine; TRE, tetradecanoyl phorbol acetate (TPA) response element.

[2] G. R. Post, L. R. Collins, E. D. Kennedy, A. M. Aragay, D. Goldstein, and J. H. Brown, submitted for publication.

al., 1993) and Rat-1 fibroblasts (Voyno-Yasenetskaya *et al.*, 1994a). In addition, epidermal growth factor-stimulated mitogen-activated protein kinase activity is enhanced in fibroblasts that express the activated mutants of $G\alpha_{12}$ and $G\alpha_{13}$ (Voyno-Yasenetskaya *et al.*, 1994b). Therefore, these G-proteins appear to be good candidates to be involved in PTX-insensitive signaling pathways leading to cell growth.

In the present study we show that the activated mutant form of $G\alpha_{12}$ induces AP-1-mediated transcriptional activation when transfected in 1321N1 cells, and this effect requires Ras function. Furthermore, we show that microinjection of an antibody against $G\alpha_{12}$ can specifically block the mitogenic effect of thrombin. Taken together, these results suggest that $G\alpha_{12}$ functions as a PTX-insensitive mediator of thrombin-induced DNA synthesis in 1321N1 cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Microinjection—1321N1 human astrocytoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Cellgrow) supplemented with 5% fetal bovine serum (Gemini) at 37 °C in an atmosphere containing 10% CO_2. For microinjection, cells were plated at 50–60% confluence on 12-mm coverslips, grown for 1 day, and rendered quiescent by serum deprivation for 24 h prior to microinjection. All microinjection experiments were performed using an automatic manipulator (Eppendorf, Fremont, CA). Microinjection needles were pulled on a vertical pipette puller (Kopf, Tujunga, CA). Affinity-purified CT95 (anti-$G\alpha_{12}$), CT112 (anti-$G\alpha_o$), and nonimmune IgG antibodies were dissolved in microinjection buffer containing 20 mM sodium phosphate and 50 mM NaCl and injected into the cytoplasm of quiescent cells (at the concentration indicated in the figure). Three hours postinjection the cells were stimulated by either 0.5 unit/ml thrombin (Sigma), 5% fetal calf serum, or 20 ng/ml basic fibroblast growth factor (bFGF) as indicated in the legends. The thymidine analog bromodeoxyuridine (BrdUrd) (Amersham Corp.) was added to the incubation medium. Twenty-four hours after stimulation the cells were fixed in 95% ethanol and 5% acetic acid and processed for immunofluorescence.

Immunofluorescence Staining—To identify injected cells, the coverslips were washed with phosphate-buffered saline containing 0.1% Tween 20 and incubated for half an hour at 37 °C with a fluorescein-conjugated anti-rabbit antibody (Cappel) at 20 μg/ml in phosphate-buffered saline containing 1 mg/ml bovine serum albumin, 0.5% Nonidet P-40. Cells that had incorporated BrdUrd into synthesized DNA were detected by indirect immunofluorescence as described (LaMorte *et al.*, 1993b). Briefly coverslips were incubated with a monoclonal anti-BrdUrd antibody (Amersham Corp.) followed with a rhodamine-conjugated anti-mouse antibody (Cappel) at 1:100 dilution. The cells were analyzed and photographed with a Zeiss Axiophot fluorescent microscope.

Antibody Purification—Antibodies against $G\alpha$ subunits G_{12} and G_{oa} (CT95 and CT112, respectively) were made in rabbits using synthetic peptides conjugated to keyhole limpet hemocyanin. The $G\alpha_{12}$ C-terminal peptide sequence used to raise the CT95 antibody is CQENLKDIMLQ, and the $G\alpha_o$ C-terminal peptide sequence (CT112) is CANNLRGCGLY. The antibodies were purified by affinity chromatography using the synthetic peptides coupled to an Affi-Gel 15 (Bio-Rad) matrix. Elution was performed with 100 mM glycine, pH 2.5. The eluants were dialyzed against 20 mM phosphate buffer, concentrated, aliquoted, and stored at −70 °C until used.

SDS-Polyacrylamide Gel Electrophoresis and Western Blot—SDS-polyacrylamide gel electrophoresis was performed as described (Laemmli, 1970). COS-7 cells were transiently transfected with the plasmids: pCISG12, pCISG13, pCISGq, or control vector pCISLacZ. Transfected COS-7 and 1321N1 membranes were prepared as described previously (Aragay *et al.*, 1992). One volume of SDS-loading buffer was added to the cell extracts, boiled for 5 min, centrifuged, and loaded onto a 12% SDS-acrylamide gel. Gels were electrotransferred to nitrocellulose membranes, immunostained with $G\alpha$ antibodies, and visualized using the ECL method (Amersham Corp.).

Transfection of 1321N1 Cells—Human 1321N1 astrocytoma cells were plated 1 day before transfection and were transfected by the calcium phosphate coprecipitation method (Trejo *et al.*, 1992). Each 60-mm plate received a total of 12 μg of DNA. Varying amounts of pCISG12Q229L or pCISGqR183C or pCDNA1GiR179C or the corresponding control vectors pCIS or pCDNAI were transfected with 4 μg of an AP-1-responsive reporter plasmid (2 × TRE) consisting of two consensus TRE sequences (recognized by the AP-1 complex) upstream of the minimal prolactin promoter linked to the luciferase reporter gene (2 × TRE-LUC). In some cases control vector was added to achieve a final DNA concentration of 12 μg per plate. For the dominant negative Ras studies, cells were cotransfected with 4 μg of 2 × TRE and 4 μg of Ala-15 Ras (Powers *et al.*, 1989) or its control vector, pZIP, and 4 μg of either pCISG12Q229L, pCISGqR183C, or pCISLacZ. 48 h later cells were lysed in 100 mM potassium phosphate, pH 7.9, containing 1% Triton X-100 and 1 mM dithiothreitol. Luciferase activity was measured in 20 μl of lysate as described previously (Trejo *et al.*, 1992).

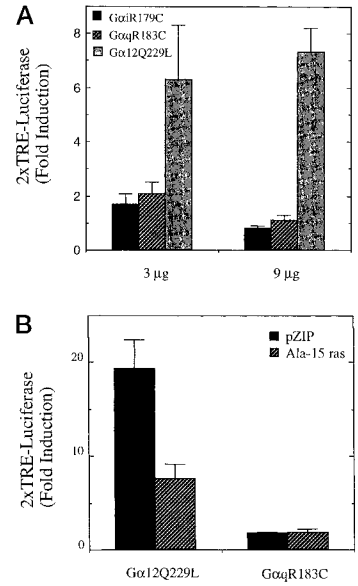

FIG. 1. **Effect of $G\alpha_{12}$ on 2 × AP-1-luciferase gene expression.** Subconfluent 1321N1 cells were transiently transfected as described under "Experimental Procedures." *A*, cells were transfected with 3 or 9 μg of either pCISGα12Q229L, pCISGαqR183C, or pCISGαiR179C or backbone vector and assayed for luciferase activity. *B*, cells were transfected with either pCISGα12Q229L or pCISGαqR183C in the presence of Ala-15 Ras or its backbone vector pZIP and harvested 48 h later. The cells were subsequently assayed for luciferase activity. The -fold induction is calculated by comparison with cells transfected with the control plasmid and normalized to total protein. Each *bar* represents the mean ± S.E. of data from three separate experiments, each containing three to four replicates.

RESULTS AND DISCUSSION

The thrombin receptor is a member of the G-protein-coupled receptor superfamily and has been shown to couple to G_i, G_o, and G_q proteins in different signaling pathways (Vu *et al.*, 1991; LaMorte *et al.*, 1993a; Baffy *et al.*, 1994). In addition, the thrombin receptor has been shown to couple to G_{12} in platelets (Offermanns *et al.*, 1994). In 1321N1 cells thrombin stimulates the DNA binding activity of the transcription complex AP-1 and results in an associated increase in AP-1-mediated gene expression (Trejo *et al.*, 1992). This response cannot be explained by activation of either G_i or G_q alone.[2] To test the possible involvement of $G\alpha_{12}$ in AP-1-mediated gene expression we examine the ability of an activated GTPase-deficient form of $G\alpha_{12}$ to transactivate an AP-1-responsive reporter plasmid (2 × TRE-LUC). 1321N1 cells were transiently co-transfected with plasmids containing the constitutively activated (GTPase-deficient) mutant forms of $G\alpha_{12}$ (Q229L), $G\alpha_q$ (R183C), or $G\alpha_i$ (R179C) or with a control plasmid along with the 2 × TRE-LUC reporter. The activated $G\alpha_{12}$ caused a 6–7-fold increase in luciferase activity relative to control transfected cells (Fig. 1*A*). Only a modest (<2-fold) stimulation was seen with the activated G_q and G_i mutants. The marked stimulatory effect of the activated $G\alpha_{12}$ mutant suggests that G_{12} protein may couple thrombin-receptor activation to the induction of the expression of AP-1-responsive target genes.

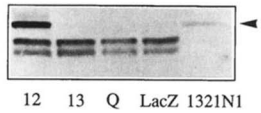

FIG. 2. **Identification of $G\alpha_{12}$ in 1321N1 astrocytoma cells by Western analysis.** Extracts of COS-7 cells transiently transfected with pCISGα12, pCISGα13, pCISGαq, and control plasmid (pCISLacZ) and 1321N1 membrane extracts were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotted with CT95 antibody.

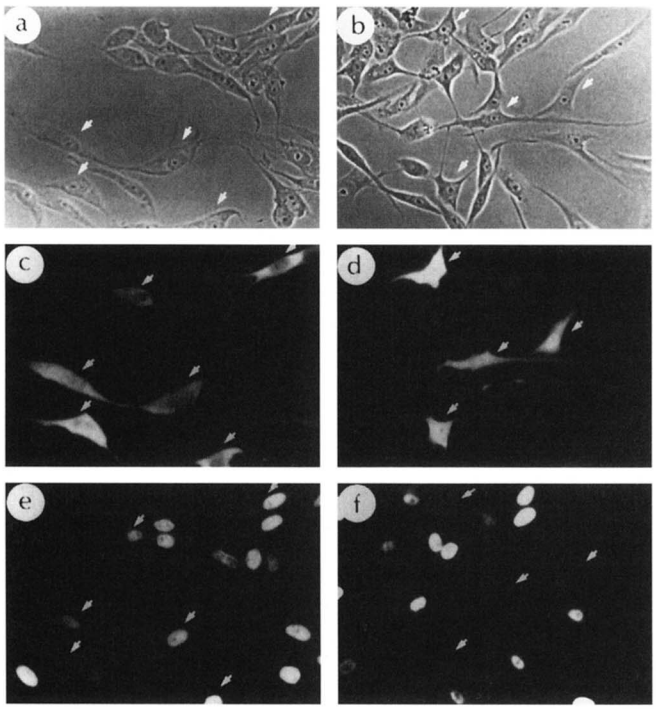

FIG. 3. **Microinjection of CT95 antibody against $G\alpha_{12}$ in thrombin-stimulated 1321N1 cells.** Cells were plated on glass coverslips and starved 24 h prior to injection. Random areas of quiescent cells were injected with control IgG (10 mg/ml) or CT95 antibody (10 mg/ml). Cells were stimulated with 0.5 unit/ml thrombin in DMEM. Representative examples of injected cells are shown in *panels a* (IgG) and *b* (CT95). *Panels c, e* and *d, f* represent the same field as *a* and *b*, respectively. *a, b*, phase contrast micrograph; *c, d*, fluorescent photomicrographs depicting injected cells; *e, f*, fluorescent photomicrographs depicting injected cells stained for BrdUrd incorporation. *Arrows* indicate the injected cells.

To examine the involvement of Ras in the stimulation of AP-1 gene expression, the Ala-15 dominant inhibitory mutant of Ras (Powers *et al.*, 1989) was coexpressed with the activated forms of either $G\alpha_{12}$ or $G\alpha_q$ and the AP-1-sensitive luciferase reporter plasmid (Fig. 1*B*). The $G\alpha_q$-induced increase in AP-1 transcriptional activity was not affected by Ala-15 Ras. However, expression of the Ala-15 Ras mutant led to a 70% reduction in the $G\alpha_{12}$-induced luciferase activity. These results demonstrate that $G\alpha_{12}$ stimulation of AP-1-dependent gene expression at least partially requires Ras. Together with our previous observation that Ras is required for thrombin stimulation of AP-1 activity and mitogenesis (LaMorte *et al.*, 1993b), these results suggest that $G\alpha_{12}$ may couple the thrombin receptor to Ras activation.

To more specifically examine the involvement of $G\alpha_{12}$ in thrombin-stimulated mitogenesis, we microinjected inhibitory $G\alpha_{12}$ antibodies into intact 1321N1 astrocytoma cells. The specificity of the antiserum was first verified by Western blot using extracts of COS-7 cells transiently transfected with plasmids encoding $G\alpha_{12}$, $G\alpha_{13}$, $G\alpha_q$, and LacZ (Fig. 2). The antiserum raised against a peptide corresponding to the 11 C-terminal amino acids of $G\alpha_{12}$ (referred to as CT95 or anti-$G\alpha_{12}$ antibody) recognized a protein of the expected size in COS-7 cells expressing $G\alpha_{12}$ but did not detect other G-protein α subunits. The CT95 antiserum also recognized a protein of the expected size in 1321N1 astrocytoma cells.[2]

Serum-deprived 1321N1 cells were microinjected with either the anti-$G\alpha_{12}$ antibody, nonimmune IgG, or anti-$G\alpha_o$ antibody (CT112). Three hours later, the cells were treated with 0.5 unit/ml thrombin. The nucleotide analog BrdUrd was added to the medium, and 24 h later the cells were fixed. Injected cells were simultaneously assessed for the presence of injected antiserum and for the nuclear incorporation of BrdUrd by immunofluorescence microscopy (Fig. 3). Thrombin stimulated DNA synthesis in 50 ± 3% of the cells while only 10 ± 2% of unstimulated cells synthesized DNA (Table I). Injection of the CT95 antibody (10 mg/ml) reduced the proportion of cells in which thrombin stimulated DNA synthesis to 23 ± 3% (Table I). There was a direct correlation between the amount of antibody injected and the degree of inhibition of BrdUrd incorporation (Fig. 4). Microinjection of 15 mg/ml CT95 antibody resulted in a complete abolition of thrombin-stimulated DNA synthesis but had no effect on basal BrdUrd incorporation in unstimulated cells. Preincubation of the CT95 antibody with the peptide immunogen resulted in loss of the ability of the anti-$G\alpha_{12}$ antibody to block thrombin-induced DNA synthesis

TABLE I
Effects of CT95(Ab12) on thrombin-stimulated DNA synthesis

Treatment	Injected material	BrdUrd incorporated in injected cells	BrdUrd incorporated in uninjected cells	Reduction
		%	%	%
Quiescent[a]			10 (n = 200)[b]	
Thrombin[c]	CT95(Ab12)[d]	21 (n = 221)	46 (n = 240)	69[e]
Thrombin	IgG	45 (n = 240)	52 (n = 331)	0
Thrombin	CT112(AbGo)	52 (n = 311)	46 (n = 269)	0
Quiescent[f]			10 ± 2[g] (n = 1130)	
Thrombin	CT95(Ab12)	23 ± 3 (n = 862)	47 ± 2 (n = 1683)	63
Thrombin	IgG	49 ± 4 (n = 719)	50 ± 3 (n = 1003)	0

[a] Values obtained from random fields injected on separate coverslips in a single-day experiment.
[b] n is the number of injected cells or, in the case of uninjected cells, the number of cells analyzed.
[c] Fully supplemented growth medium containing 0.5 unit/ml of thrombin.
[d] CT95, CT112, and IgG were injected at 10 mg/ml.
[e] Percent reduction corresponds to percent of DNA synthesis (uninjected) minus percent of DNA synthesis (injected) divided by the percent of DNA synthesis (uninjected) after the background has been subtracted.
[f] The values obtained from the analysis of random fields injected on separate coverslips in seven separated experiments. A C test of independence show significant difference ($p >> 0.001$) between CT95 injected and control injected or uninjected cells.
[g] Standard error of proportion.

(data not shown). There was no inhibition of the response to thrombin in cells injected with equivalent concentrations of nonimmune IgG. Furthermore, microinjection of an anti-$G\alpha_o$ did not reduce the number of cells undergoing DNA synthesis in response to thrombin (Table I). These results clearly demonstrate that inhibitory antibodies directed against $G\alpha_{12}$ can specifically block thrombin-induced DNA synthesis in 1321N1 cells.

To further demonstrate the specificity of the blocking effect of the CT95 antibody we examined the effect of this antibody on induction of DNA synthesis by bFGF. The actions of bFGF are presumed to be mediated through tyrosine phosphorylation and do not utilize G-protein signaling pathways. Quiescent 1321N1 cells were injected with either the anti-$G\alpha_{12}$ antibody or control nonimmune IgG and subsequently stimulated with 20 ng/ml bFGF or with 0.5 unit/ml thrombin. bFGF induced DNA synthesis in 22 ± 4% of the cells (Table II). Microinjection of the anti-$G\alpha_{12}$ antibody (10 mg/ml) did not inhibit the bFGF-stimulated DNA synthesis. These results indicate that bFGF does not utilize $G\alpha_{12}$ for mitogenic signaling and that the anti-G_{12} antibody does not exert a generalized inhibitory effect upon DNA synthesis. These data further strengthen the argument for the specificity of action of the anti-G_{12} antibody on the response to thrombin.

We additionally examined the effect of the CT95 antibody on the serum stimulation of DNA synthesis. The addition of 5% fetal calf serum to serum-deprived 1321N1 cells stimulated DNA synthesis in 76–90% of the cells (Table II). Microinjection of the anti-$G\alpha_{12}$ antibody reduced the response to serum by 45%. A similar effect was likewise observed by LaMorte *et al.* (1992) with an anti-$G\alpha_i$ antibody in serum-stimulated fibroblasts where the thrombin response is PTX-sensitive. The inhibitory effect of the anti-$G\alpha_{12}$ antibody suggests that serum-induced DNA synthesis in 1321N1 cells is mediated in part through receptor(s) that function via $G\alpha_{12}$. This observation argues that G_{12} is a necessary cellular transducer for certain types of mitogens in addition to thrombin.

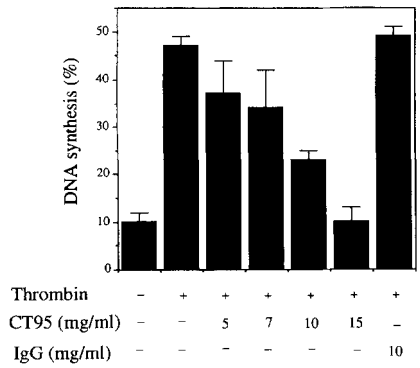

FIG. 4. **Percent of DNA synthesis in thrombin-stimulated 1321N1 cells injected with CT95 antibody.** Cells were plated on glass coverslips and starved 24 h prior to injection. Quiescent cells were injected in random fields with CT95 and IgG at the concentrations indicated in the *lower panel*. Cells were stimulated with 0.5 unit/ml thrombin in DMEM. DNA synthesis was statistically analyzed using the standard error of proportion comparing injected cells and uninjected cells from the same coverslips. Fields of 200-1200 cells were counted for each point. The results presented represent the means of at least two experiments. *Error bars* represent the 95% confidence interval calculated by using the standard error of proportion.

Our previous work[2] demonstrated that the mitogenic response to thrombin in 1321N1 cells was pertussis toxin-insensitive, and we suggested that an additional pathway besides the G_q/phospholipase C pathway was required. The results presented here suggest that G_{12} is the other transducer mediating the mitogenic effect of thrombin in the 1321N1 cells. The inhibition of thrombin-stimulated DNA synthesis by the G_{12} antibody coupled with the activation by G_{12} of AP-1-dependent transcription reported here provides the first evidence of a signaling pathway in which G_{12} links a G-protein receptor to cell growth. It also suggests a new linkage between a G-protein pathway and the oncogene *ras*.

The mechanism by which G_{12} affects cell growth is still not clear. Recent data demonstrate that $\beta\gamma$ released from stimulation of G_i-coupled receptors can mediate the activation of MAP kinases acting on a Ras-dependent pathway (Winitz *et al.*, 1993; Albas *et al.*, 1993; Faure *et al.*, 1994; Crespo *et al.*, 1994; Koch *et al.*, 1994). $\beta\gamma$ released from G_{12} by thrombin activation could mediate the mitogenic response. However, the experiments reported here demonstrate that the α subunit of G_{12} alone is able to induce AP-1-mediated gene expression. Other studies show that $G\alpha_{12}$ or the constitutively activated $G\alpha_{12}$ subunits are able to cause cell transformation (Chan *et al.*, 1993; Jiang *et al.*, 1993). It seems likely therefore that there is a pathway activated by the α subunit of G_{12}, which can lead to cell growth in some systems.

The direct effector of $G\alpha_{12}$ is not known. Responses shown to be indirectly mediated via G_{12} are the activation of phospholipase A_2 (Xu *et al.*, 1993) and of a Na^+-H^+ exchanger (Dhanasekaran *et al.*, 1994), which is also regulated by G_{13} (Voyno-Yasenetskaya *et al.*, 1994c). Since the activation of the Na^+-H^+ exchanger involves protein kinase C (Dhanasekaran *et al.*, 1994) it is possible that the activation of a particular isoform of protein kinase C by the G_{12} pathway may contribute to the mitogenic effect. In addition, $G\alpha_{12}$-mediated gene expression is at least partially Ras-dependent, and thus $G\alpha_{12}$ may regulate

TABLE II
Effects of CT95(Ab12) on thrombin, serum, and fibroblast growth factor (FGF) stimulated DNA synthesis in 1321N1 cells

Treatment	Injected material	BrdUrd incorporated in injected cells	BrdUrd incorporated in uninjected cells	Reduction
		%	%	%
Quiescent			6 ± 2 (n = 657)	
Thrombin[a]	CT95(Ab12)[b]	21 ± 6[c] (n = 201)[d]	46 ± 4 (n = 507)	54[e]
Thrombin	IgG	50 ± 8 (n = 147)	48 ± 6 (n = 217)	0
Serum[f]	CT95(Ab12)	48 ± 3 (n = 514)	87 ± 3 (n = 365)	45
Serum	IgG	83 ± 8 (n = 78)	81 ± 5 (n = 231)	0
FGF[g]	CT95(Ab12)	21 ± 7 (n = 110)	22 ± 4 (n = 323)	0

[a] Fully supplemented growth medium containing 0.5 unit/ml of thrombin.
[b] CT95 and IgG was injected at 10 mg/ml.
[c] Standard error of proportion.
[d] n is the number of injected cells, or in the case of uninjected cells, the number of cells analyzed.
[e] Percent reduction corresponds to percent of DNA synthesis (uninjected) minus percent of DNA synthesis (injected) divided by percent of DNA synthesis (uninjected) after the background has been subtracted.
[f] Fully supplemented growth medium containing 5% fetal calf serum.
[g] Fully supplemented growth medium containing 20 ng/ml bFGF.

proteins responsible for Ras activation. Further work will be necessary to clarify the G_{12} effector pathway and the steps leading to mitogenesis.

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